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Polyribosomes Associated with Synaptic Specializations on Axon Initial Segments: Localization of Protein-Synthetic Machinery at Inhibitory Synapses

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Previous studies have revealed a selective association between polyribosomes and axospinous synapses in a variety of brain regions. The present study evaluates whether polyribosomes are also associated with the symmetrical and presumably inhibitory synaptic connections found on the initial segment of axons of some neurons in the CNS. The initial segments of pyramidal neurons in the sensorimotor cortex of the monkey and of granule cells in the hippocampus of the rat were examined. The initial segments of these cell types are contacted by GABAergic terminals that form symmetrical synaptic connections. In the present study, these initial segments were found to contain polyribosomes that tended to be selectively localized beneath the synaptic specializations. Both the synaptic connections and the polyribosomes were localized to the initial segment; after the point at which the axon became myelinated, neither synapses nor polyribosomes were found. The association between polyribosomes and synapses was also suggested by the position of the polyribosomes with respect to the synapse. In each cell type, the majority of the polyribosomes that were present in the initial segments appeared to be localized preferentially beneath synaptic sites, although some polyribosomes were also present in the core of the axon. These data reveal that polyribosomes are not peculiar to spine synapses, but rather are ubiquitous components of the subsynaptic region of many types of synapses. We propose that neurons may regulate their innervation by positioning protein-synthetic machinery (and appropriate mRNA molecules) at particular locations in order to construct particular types of synapses at defined positions on the postsynaptic cells' receptive surface.

Since the earliest electron-microscopic identification of ribosomes as the site of protein synthesis, it has been known that most of the protein-synthetic machinery of the neuron is localized in the soma and the most proximal portions of the dendrites (Palay and Palade, 1955). Exceptions to this generalization were recognized, but no particular functional significance was attached to these exceptions. For example, dendrites were known to contain a few clusters of polyribosomes throughout their length, and some polyribosomes were also reported in axon initial segments (see Peters et al., 1976). These clusters were generally thought to be randomly scattered, except for one report that showed some axonal ribosomes grouped at points of branching and beneath synaptic contacts (Jones and Powell, 1969). Therefore, they were usually considered, only in passing, as exceptions to the rule. Thus, it has come to be accepted as a central tenet that essentially all of the protein constituents of the neuron, including those required for synaptic specializations, were synthesized in the cell body, and somehow selectively transported to the sites at which they would be assembled (for a review, see Grafstein and Forman, 1980).

Over the past few years, a series of observations has given rise to an hypothesis that challenges the prevailing view that virtually all neuronal proteins are produced in the cell body and transported to their eventual sites of use. This hypothesis arose from studies that revealed that most of the polyribosomes in dendrites were not simply scattered in the dendritic cytoplasm, but rather were selectively positioned at the base of dendritic spines (Steward, 1983b; Steward and Levy, 1982). These observations suggested that some protein(s) were synthesized in discrete cytoplasmic microdomains associated with individual synaptic sites on spines (Steward, 1983b; Steward and Levy, 1982).

There has been growing evidence that the polyribosomes under synaptic sites play an important role during synapse growth or modification. For example, during periods of lesion-induced synaptogenesis, the incidence of polyribosomes under spine synapses increased 3-fold in areas being reinnervated (Steward, 1983a). The increases in the incidence of polyribosomes under synaptic contacts were paralleled by increases in protein synthesis in the denervated neuropil (Fass and Steward, 1983; Steward and Fass, 1983). Furthermore, studies in developing animals have revealed a relationship between growing synapses and polyribosomes (Steward and Falk, 1985, 1986). The proportion of synapses with underlying polyribosomes was about 6-fold higher in developing animals than at maturity. The prominence of the polyribosomes during synapse growth suggested that local synthesis of protein at the synaptic site might be particularly important during synapse construction.

These earlier studies left several questions unanswered. First, virtually all of the observations involved asymmetric (Gray's type I) synapses that contacted dendritic spines; it was not known whether polyribosomes were also found in association with symmetric (Gray's type II) synapses, or with non-spine synapses. A second, related question was whether the polyribosomes were associated only with excitatory synapses or with excitatory and inhibitory synapses. To explore these issues, we undertook an analysis of synaptic contacts on axon initial segments. The best characterized of these is found upon the initial segments of axons of pyramidal cells of the cerebral cortex and hippocampus. In the cerebral cortex, many, if not all, of the terminals innervating the initial segments arise from a specialized type of local circuit neuron, the chandelier cell (DeFelipe et al., 1985; Freund et al.,

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1983; Peters et al., 1982; Somogyi, 1977; Somogyi et al., 1982). These terminals form symmetric synaptic junctions. In addition, many of the terminals are immunoreactive for glutamic acid decarboxylase (GAD), which suggests that they are inhibitory (see DeFelipe et al., 1985; Freund et al., 1983; Kosaka et al., 1984; Peters et al., 1982; Ribak, 1978, 1985; and Somogyi et al., 1985). Similar types of synaptic connections with axon initial segments are also found in the hippocampal formation (Kosaka et al., 1984; Somogyi et al., 1983). Since these synapses on axon initial segments are non-spine, symmetric, and presumably inhibitory, an opportunity is provided to address both of the issues raised above. In the present report, we describe an association between polyribosomes and synaptic sites on axon initial segments in the cerebral cortex and hippocampal formation.

Materials and Methods

The present observations were made for the most part using a collection of materials originally prepared for other purposes. Specimens from the monkey sensory motor cortex were obtained from the control hemisphere contralateral to an epileptic focus created by the application of alumina gel (Ribak et al., 1982). These animals were fixed by intracardiac perfusion with mixed aldehydes (4% paraformaldehyde/0.1% glutaraldehyde, 0.002% $CaCl_2$ in 0.12 M phosphate buffer). Specimens from the hippocampus of adult Sprague-Dawley rats were obtained following intracardiac perfusion with 2% paraformaldehyde/2% glutaraldehyde in cacodylate buffer (Steward and Falk, 1985, 1986; Steward and Levy, 1982).

Thick sections of the chosen brain regions were cut at 50–300 μm, either with a Sorvall tissue chopper or an Oxford Vibratome. Sections from the cerebral cortex were cut parallel to the long axis of the pyramidal neurons in order to obtain sections that yielded the longest segments of apical dendrites; such sections usually had the largest number of identified axon initial segments (Ribak, 1985). Coronal sections from the dorsal portion of the hippocampus yielded sections oriented more or less parallel with the long axis of the resident neurons. Blocks from the chosen regions were trimmed by hand, osmicated, and embedded in plastic, using routine protocols. Semithin (1 μm) sections were stained with toluidine blue to aid in orientation, and thin sections were stained with uranyl acetate/lead citrate and mounted on Formvar-coated slot grids prior to examination with an electron microscope.

As in previous studies (Steward, 1983a, b; Steward and Falk, 1985, 1986; Steward and Levy, 1982), polyribosomes have been identified as collections of electron-dense particles the size of ribosomes, exhibiting an interparticle spacing typical of ribosomal rosettes. The uniform size and staining properties of these particles make them easily distinguishable from other known elements, including glycogen. Nevertheless, this identification is of course a morphological one, and it is possible that some of the elements identified as polyribosomes are some unidentified cytoplasmic entities. In the absence of any evidence to the contrary, however, these particles are termed ribosomes without qualification in the present study. Synaptic contacts were considered positive for polyribosomes if at least 3 ribosomes were present in the cluster, and if the cluster was located directly under the synaptic specialization without any intervening organelles.

Results

Synaptic contacts on axon initial segments have been described in previous studies for each of the brain regions under consideration. Only a brief review is possible here, the focus being on the subsynaptic cytoplasm, particularly the distribution of polyribosomes. The present analysis concentrates on the cerebral cortex, because the synapses on initial segments of cortical pyramidal cells are better characterized than those of other brain regions (see above and, for a review, Peters, 1984).

Sensorimotor cortex of monkeys

The observations of the present study were limited to the axon initial segments of pyramidal cells in layers II, III, and V. The ultrastructural features of these neurons have been described previously (Ribak, 1985; Ribak et al., 1982). In brief, pyramidal neurons have multiangular-shaped somata formed by an array of basal dendrites, a single apical dendrite, and a single axon usually located between the basal dendrites. The soma contains a relatively large, round nucleus, which is centrally located. Numerous organelles are found in the perikaryal cytoplasm; they include mitochondria, polyribosomes, and cisternae of granular endoplasmic reticulum (Fig. 1*A*).

Axon initial segments arise at the base of the pyramidal cell soma from an axon hillock region that is usually sparsely populated with polyribosomes and cisternae of granular endoplasmic reticulum. Thus, at the light-microscopic level, the axon hillock area of the cell body appears lucent, and lacks Nissl bodies. The conical-shaped axon hillock tapers into the initial segment, and bundles of microtubules extend through these 2 regions.

Axon initial segments usually have a cylindrical shape, and are of a constant diameter of about 1 μm throughout their length (Figs. 1, *A* and *B*, and 2). The most distal part of the initial segment becomes myelinated, and is characterized by a typical paranodal region (Fig. 2*A*). Initial segments contain bundles of microtubules, in contrast to most dendrites, in which the microtubules are well spaced. The most distinctive characteristic of the initial segment is the dense undercoating of the axolemma (Figs. 1 and 2). The undercoating permits an identification of isolated profiles of initial segments even when the segments are not continuous with a cell body or a myelinated segment. Other organelles frequently observed in initial segments include cisternae and vesicles of agranular reticulum, neurofilaments, elongated mitochondria, occasional polyribosomes, and cisternal organelles that resemble a spine apparatus (see Fig. 1*B*). Numerous terminals with pleomorphic vesicles form symmetric synapses throughout the entire length of the initial segment (DeFelipe et al., 1985).

We observed that polyribosomes were infrequent in the axon hillock region. By contrast, the proximal axon initial segment displayed numerous polyribosomes (Fig. 1, *A–C*), most of which were found in the periphery of the axis cylinder. Many of the clusters of polyribosomes were positioned directly under the synaptic specializations described above. When cisternal organelles were present beneath a synapse, polyribosomes were often found between the organelle and the axolemma (Fig. 1*B*). Qualitative observations strongly suggested selectivity of the polyribosome localization, in that most of the polyribosomes appeared to be associated with synaptic specializations.

While many of the polyribosomes were positioned directly under synaptic specializations, some were not obviously associated with a synaptic site. In some instances, the polyribosomes appeared adjacent to a terminal that did not make a morphologically identifiable contact in the individual section under consideration, as is shown in Figure 1*B*, terminal 2. For this particular contact, examination of adjacent sections revealed that terminal 2 did contact the initial segment, and thus the polyribosomes are clearly in a perisynaptic region in this case. A few clusters of polyribosomes were centrally located within the axis cylinder, however (Fig. 2*A*). These clusters were often as-

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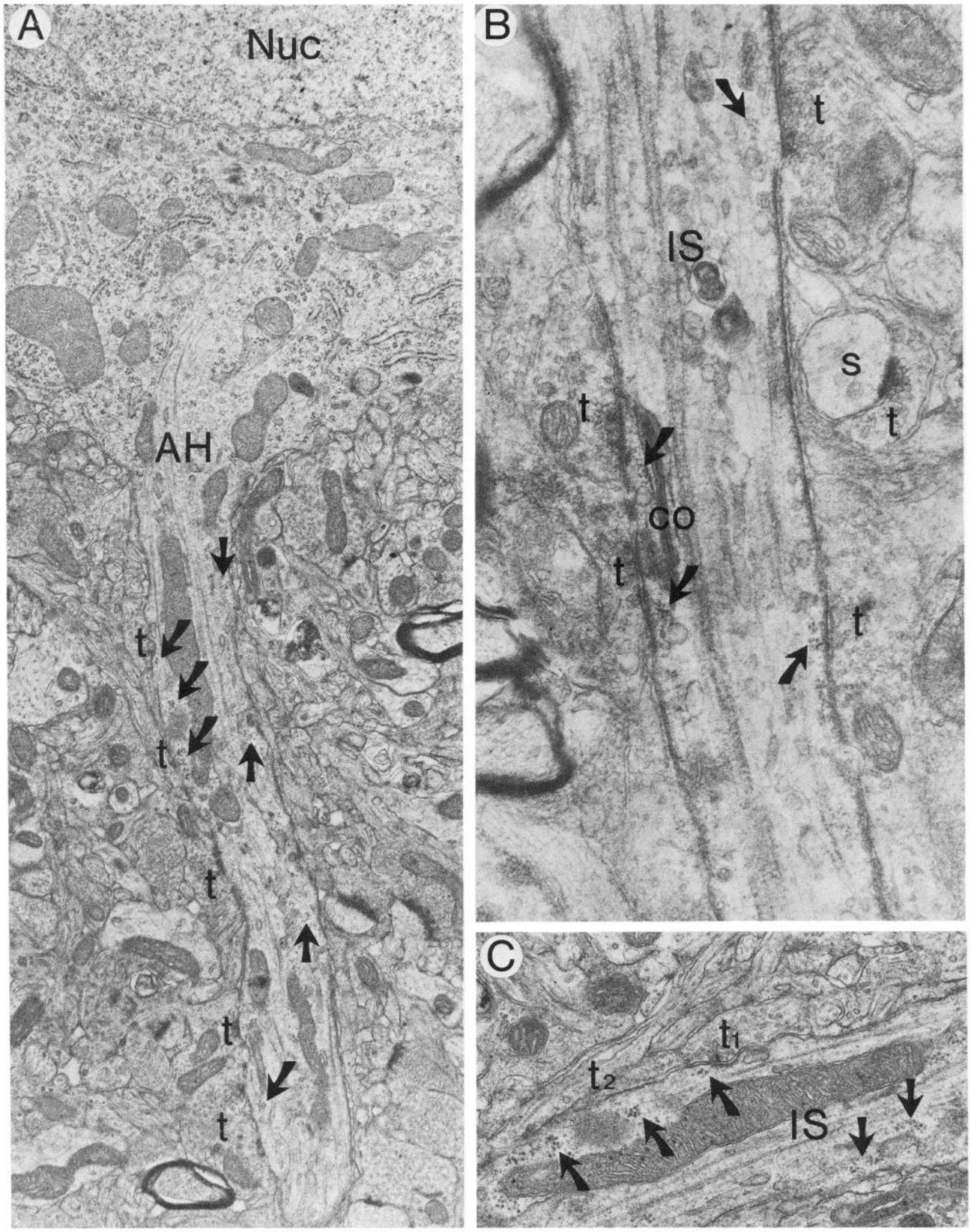
Figure 1. Axon initial segment of pyramidal neurons in monkey sensorimotor cortex. *A*, Cell body, axon hillock (*AH*), and initial segment of a layer III pyramidal cell. The nucleus (*Nuc*) is partially visible, as well as numerous cisternae of granular endoplasmic reticulum within the cell body. The axon hillock is relatively devoid of rough endoplasmic reticulum or free polyribosomes. Terminals (*t*) can be seen contacting the axon initial segment, which is marked by the typical dense undercoating; polyribosomes can be seen beneath many of these synaptic contacts (*curved arrows*). *Straight arrows* indicate polyribosomes that do not appear to be associated with synaptic contacts. A portion of this initial segment is



illustrated at higher power in *C*. ×17,000. *B*, A portion of an axon initial segment (*IS*) which features the typical dense undercoating of the axolemma, fasciculation of microtubules, and a cisternal organelle (*CO*). Terminals (*t*) form symmetric synapses with this axon, and polyribosomes (*arrows*) are found under many of these junctions. A neighboring terminal (*t*) forms an asymmetric synapse with a spine (*s*). (Photograph courtesy of Dr. J. DeFelipe.) ×37,000. *C*, At higher power, a portion of the axon initial segment illustrated in *A*. Note that both the terminal that forms a symmetric synapse in this section (*t1*) and the nearby terminal that apparently does not form a synapse (*t2*) have polyribosomes (*curved arrows*) beneath the site on the membrane apposed to the terminal. Examination of adjacent sections revealed that *t2* also formed a synaptic contact on the axon initial segment. *Straight arrows* indicate polyribosomes that are not apparently associated with IS synapses. ×27,000.

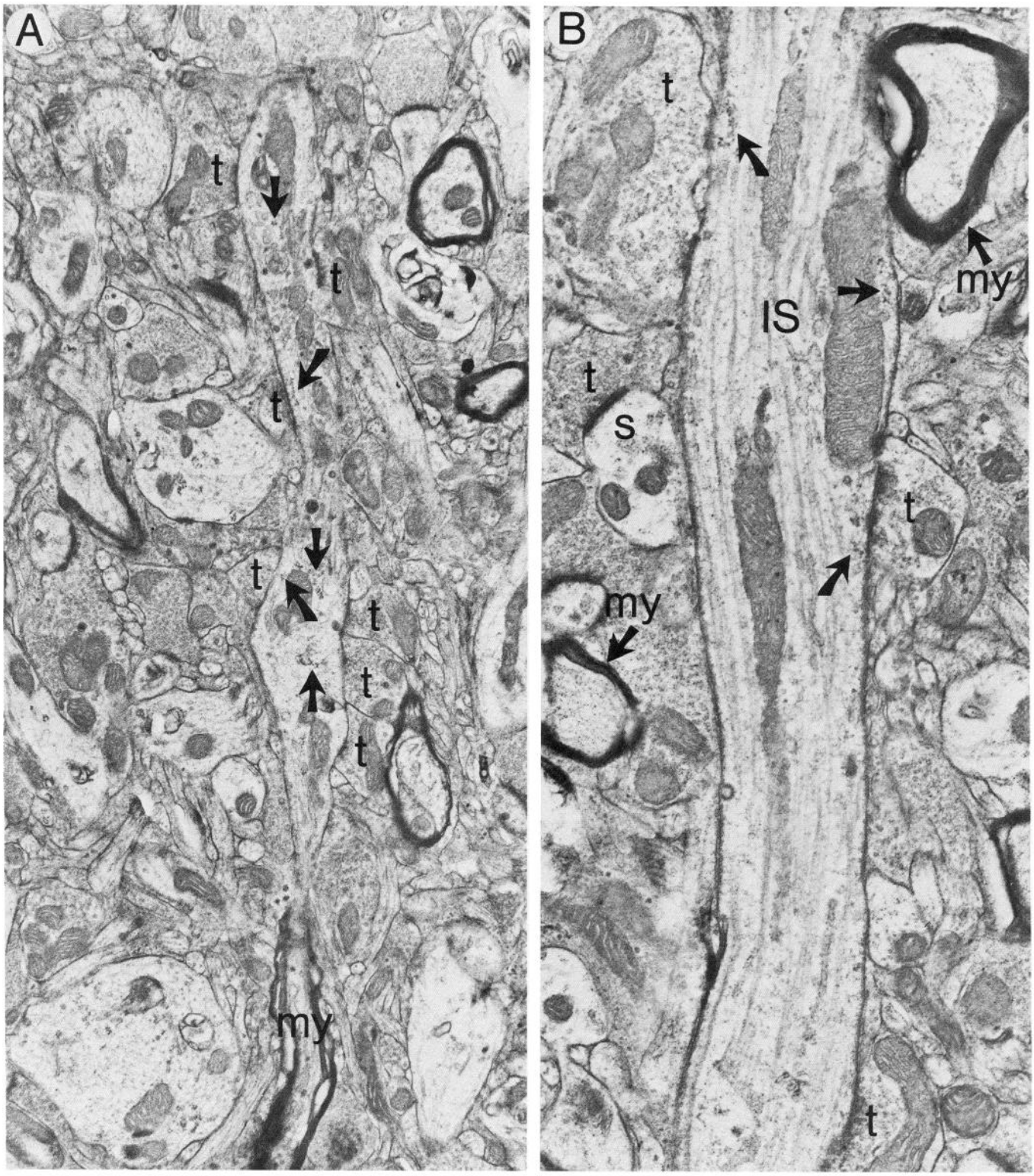


Figure 2. *A,* Distal portion of an axon initial segment of a pyramidal cell in the monkey sensorimotor cortex. Numerous terminals (*t*) contact this axon, forming symmetric synapses. Polyribosomes can be seen under some of the synaptic sites (*curved arrows*), and a few clusters of ribosomes are also visible in the core of the axon (*straight arrows*). No polyribosomes are observed in the distal myelinated portion of the axon (*my*). ×14,500. *B,* Another example of the distal portion of an axon initial segment from the sensorimotor cortex. The dense undercoating is particularly apparent. Polyribosomes (*curved arrows*) can be seen beneath 2 of the 3 synaptic contacts on this initial segment. Polyribosomes were not present in the myelinated segments of axons (*my*). An adjacent dendrite is contacted by a terminal (*t*) forming an asymmetric synapse with a stubby spine (*s*). ×25,500.

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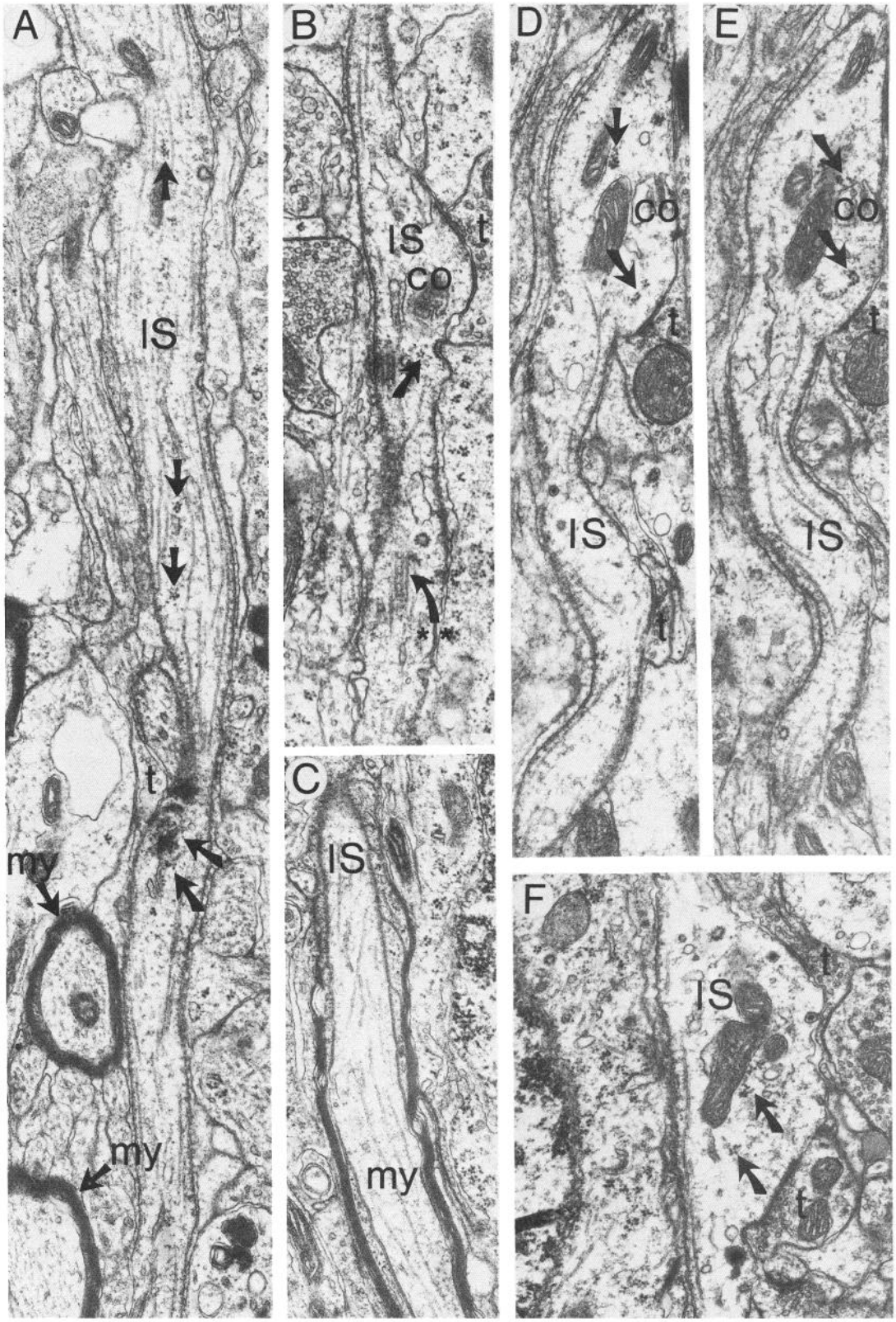
Figure 3. Axon initial segments of granule cells of the hippocampal formation of the rat. *A,* An axon initial segment (*IS*) that is relatively free of synaptic contacts. Only one potential contact is visible (under *t*), where there are prominent clusters of polyribosomes (*curved arrows*). Myelinated



axons (*my*) cut in cross section are also visible. In the regions that apparently receive no synaptic contacts, polyribosomes (*straight arrows*) tend to be clustered in the center of the axis cylinder rather than under the axolemma. *B,* An axon initial segment (*IS*) containing a cisternal organelle (*CO*) under a synaptic specialization. Polyribosomes are found near this synaptic contact. The size and staining intensity of the polyribosomes in the initial segment may be compared with the polyribosomal rosettes in the nearby cell body. The *curved arrow with asterisks* indicates a fascicle of microtubules. *C,* The transition between an initial segment (*IS*) and the myelinated portion of the axon (*my*). Note the absence of polyribosomes. *D* and *E,* An axon initial segment (*IS*) from 2 near-adjacent sections (actual distance between sections is not known). Note the cisternal organelle (*CO*) and polyribosomes (*curved arrows*) under the synapse formed by a terminal (*t*). *F,* Note that the terminals (*t*) synapse with a bulbous portion of the initial segment. Polyribosomes (*curved arrows*) are prominent in this enlarged portion of the initial segment. ×24,000.

sociated with mitochondria. Thus, while there appears to be selectivity in the association between polyribosomes and synaptic sites, the selectivity is not absolute. As previously noted, this is also the case in dendrites (Steward and Levy, 1982).

While polyribosomes were common in the initial segment, they were not observed in the myelinated portion of the axon. Similarly, no synapses were observed on the myelinated portion of the axon.

Hippocampal formation of the rat

The morphology of the initial segments of axons of hippocampal pyramidal cells and dentate granule cells was essentially comparable to that of the pyramidal cells of the monkey cerebral cortex. The undercoating of the axolemma proved to be the best marker, although fascicles of microtubules were also encountered (see Fig. 3*B*). The synaptic contacts on the initial segments of dentate granule cells were slightly different from those most commonly seen in the cortex, in that there was often an irregular mound at the synaptic site (see Fig. 3, *B, D–F*). These mounds contained a flocculent material similar to that found in dendritic spines. Cisternal organelles were often found at the base of such mounds (see Fig. 3, *B, D, E*).

As was also the case in the cerebral cortex, polyribosomes were common in initial segments and completely absent from the myelinated portion of the axon (see, for example, Fig. 3*C*). The polyribosomes tended to be localized under the synaptic sites, or near the periphery of the initial segment if there were synaptic contacts nearby. A few initial segments were encountered that lacked synapses for some distance (see Fig. 3*A*). In such segments, there were polyribosomes, but they were not concentrated at the periphery of the process.

Discussion

The principal finding of the present study—that polyribosomes are present under synaptic specializations on axon initial segments—indicates that accumulations of polyribosomes are not unique to a single structural or functional class of synapse. Previous studies revealed a selective association between polyribosomes and spine synapses in many different brain regions (Steward, 1983b), but spine synapses represent a single structural type, and most are thought to be excitatory. In contrast, the synaptic contacts with axon initial segments are non-spine, at least in the sense of what is normally considered a spine. While some of the synapses on initial segments were found on irregular mounds, not all of them were; indeed, in the cerebral cortex of the monkey, polyribosomes were found under synapses that were not associated with any irregularity in the axis cylinder. The synapses on initial segments are also symmetric, in contrast to the synapses on dendritic spines, which are usually asymmetric. In addition, in the case of the contacts made by axons of chandelier cells upon the initial segments of cortical pyramidal cells, and the contacts on pyramidal and granule cells of the hippocampus, many of the synapses are inhibitory, as evidenced by the presence of GABA and GAD immunoreactivity in the presynaptic terminal (DeFelipe et al., 1985; Freund et al., 1983; Kosaka et al., 1984; Peters et al., 1982; Ribak, 1978, 1985; Somogyi et al., 1985). Thus, the present observations, taken together with previous observations, suggest that polyribosomes may be an ubiquitous component of many types of synapses. One implication of the present study is that the polyribosomes in axon initial segments, like their counterparts in dendrites, are not accidental, but rather reflect the fact that the axon initial segment serves as a postsynaptic site, whereas the remainder of the axon usually does not.

While the present observations clearly reveal a selective localization of polyribosomes under synaptic sites on axon initial segments, some clusters of polyribosomes are not obviously associated with synaptic specializations. It is clear that some of these are in fact associated with a synaptic site that does not happen to lie within the plane of the section under consideration (for example, terminal 2 in Fig. 1*C*). On the other hand, a few clusters are located in the core of the axon, far from the axolemma. Thus, the selectivity of the association between polyribosomes and synaptic sites is not absolute; this is also true of the polyribosomes in dendrites (Steward and Levy, 1982). It is possible that some of the polyribosomes in the core of the axon have nothing to do with synapses. On the other hand, the polyribosomes under synaptic sites must reach the sites somehow, and some of the clusters that are not beneath a site at the time of fixation may be in transit or in the process of being degraded. At the moment, this issue is open to speculation, since nothing is known about how the polyribosomes and their associated mRNA are transported to synaptic sites on either axon initial segments or dendrites.

Because synapse-associated polyribosomes are particularly prominent during times of synapse growth (Steward, 1983a, b; Steward and Falk, 1985, 1986), we have argued that they produce proteins that are important for the formation of the synaptic site (Steward and Falk, 1985, 1986). The present observations provide new hints about what types of proteins might be synthesized at the subsynaptic site. The presence of protein-synthetic machinery at non-spine synapses argues against one of the possibilities suggested by our original studies (Steward, 1983b; Steward and Levy, 1982), viz., that the polyribosomes synthesize proteins for the construction or maintenance of the spine microstructure. The ubiquity of polyribosomes makes it more likely that they are involved in the production of classes of proteins common to synaptic junctions of all types. This does not necessarily imply that exactly the same proteins are produced at all synaptic sites, however. Rather, the polyribosomes may produce molecules that are unique to particular synaptic types, but that are members of classes of molecules required by all synapses. Examples include recognition molecules, neurotransmitter receptors, ion channels, or specific second-messenger generating systems.

Almost certainly, the positioning of polyribosomes under synaptic contacts must be precisely regulated. In general, the selective localization of the polyribosomes could be regulated by afferent innervation or by the postsynaptic cell. The obvious, if imperfect, way to address this question is to determine the effect of chronic deafferentation. Such an experiment has not been possible in the case of polyribosomes under dendritic spines, since in the cases that have been examined, the dendrites are rapidly and extensively reinnervated (Steward, 1983a). Indeed, as noted above, the evidence suggests that the polyribosomes might play some role in the reinnervation process. Interestingly, in alumina-gel epileptic foci, axon initial segments can be found without their normal complement of synapses; nevertheless, these denervated initial segments still contain polyribosomes (see Fig. 7 in Ribak, 1985). The presence of polyribosomes in initial segments deprived of their normal innervation suggests that the distribution of polyribosomes within neuronal processes is not directly regulated by afferent innervation.

The present data, together with results of previous studies, suggest that polyribosomes are found beneath a wide variety of postsynaptic sites, wherever these sites occur on the postsynaptic cell's receptive surface. The selective positioning of protein-synthetic machinery beneath synaptic sites invites speculation that the postsynaptic cell might regulate the construction or consolidation of particular types of synapses, in particular locations, through the local synthesis of proteins at the synaptic site. Thus, mRNA molecules coding for proteins required for synapses on axon initial segments (along with their attendant ribosomes) would be transported selectively to initial segments; mRNA molecules coding for proteins required for synapses on dendrites would be transported into dendrites. There might also

be a mechanism for distributing different classes of mRNA molecules to different proximo-distal locations on dendrites. Once in position, this machinery could synthesize the molecules necessary for the formation of the particular synaptic contact. It is not likely that all of the proteins comprising the synaptic junction would be produced in this way, but key proteins might be, with others being available in excess. By delivering the synthetic machinery for particular proteins to the synaptic site, the formidable sorting tasks of the neuron might be considerably simplified.

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